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Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1.5 Å resolution

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Lipopolysaccharide (LPS), or endotoxin, is the major mediator of septic shock, a serious complication of Gram-negative bacterial infections in humans. Molecules that bind LPS and neutralize its biological effects or enhance its clearance could have important clinical applications. *Limulus* anti-LPS factor (LALF) binds LPS tightly, and, in animal models, reduces mortality when administered before or after LPS challenge or bacterial infection. Here we present the high resolution structure of a recombinant LALF. It has a single domain consisting of three α-helices packed against a four-stranded β-sheet. The wedge-shaped molecule has a striking charge distribution and amphipathicity that suggest how it can insert into membranes. The binding site for LPS probably involves an extended amphipathic loop, and we propose that two mammalian LPS-binding proteins will have a similar loop. The amphipathic loop structure may be used in the design of molecules with therapeutic properties against septic shock.

Key words: endotoxin-neutralizing protein/horseshoe crab/membrane insertion/septic shock/X-ray structure

Introduction

Horseshoe crabs (*Limulus polyphemus* and *Tachypleus tridentatus*) are ancient arachnids that possess a primitive circulatory system, the hemolymph, containing only one kind of cell, the hemocyte. Exposure of hemocytes to bacterial endotoxins [lipopolysaccharide (LPS)] results in the activation of an intracellular coagulation cascade (Iwanaga *et al.*, 1986), a defense against microbial invasion (Nachum *et al.*, 1979). The system consists of several proteins, including one that may inhibit the cascade, called anti-LPS factor (Morita *et al.*, 1985). *Limulus* anti-LPS factor (LALF) is a small (101 amino acids), basic protein (Aketagawa *et al.*, 1986; Muta *et al.*, 1987), which binds and neutralizes LPS (Wainwright *et al.*, 1990) and has a strong anti-bacterial effect on the growth of Gram-negative R-type bacteria (Morita *et al.*, 1985).

In humans, LPS released during infection by Gram-negative bacteria can cause the severe pathological changes associated with septic shock (Duma, 1985; Glauser *et al.*, 1991). In the US, septic shock is responsible for ~100 000 deaths annually (Parrillo, 1990) and no specific drugs are available. LPS is the principal component of the outer leaflet of the outer membrane of Gram-negative bacteria (Raetz, 1990). Lipid A, the membrane anchor of LPS, consists of a central phosphodisaccharide unit that is attached to up to seven fatty acid chains and it possesses most of the biological activities of LPS (Galanos *et al.*, 1985). The toxicity in humans arises from the interaction of LPS or lipid A with membrane-bound receptors (Couturier *et al.*, 1991) or serum proteins, including the septins (Wright *et al.*, 1992) and 'lipopolysaccharide-binding protein' (LBP) (Schumann *et al.*, 1990), leading to an increase in the pro-inflammatory mediators (e.g. tumor necrosis factor, interleukin-1 and interleukin-6).

A variety of agents have been evaluated for neutralizing or enhancing the clearance of LPS *in vivo*, including the polymyxins (Morrison and Jacobs, 1976), polyclonal (Ziegler *et al.*, 1982) or monoclonal antibodies to lipid A (Ziegler *et al.*, 1991), 'bactericidal/permeability-increasing protein' (BPI) (Marra *et al.*, 1992) and LALF. Natural and recombinant LALF have been tested on various mammalian target cells and in animal models of septic shock: LALF inhibits endotoxin-mediated activation of cultured endothelial cells (Desch *et al.*, 1989) and B cells (Warren *et al.*, 1992); in animals, LALF reduces mortality when administered before or after LPS challenge (Alpert *et al.*, 1992; Warren *et al.*, 1992) or Gram-negative bacterial infection (Kupperman *et al.*, 1992). Our interest in determining the crystal structure of LALF arose from its potential in designing molecules that would have therapeutic properties in humans.

Here we present the three-dimensional structure of a recombinant LALF and suggest that an amphipathic loop on LALF represents an LPS-binding motif shared by two mammalian proteins, LBP and BPI. It has been proposed that LALF has sequence similarity with α-lactalbumin, a protein that binds LPS *in vitro* (Aketagawa *et al.*, 1986), but our results show that no structural similarity exists.

Results and discussion

Crystallography

The crystal structure of a recombinant LALF was determined by multiple isomorphous replacement using two heavy atom derivatives. An electron density map calculated at 2.0 Å resolution was immediately interpretable and a complete model including side-chains was built starting at the second residue of the authentic protein and finishing at the C-terminus. The model, including 150 water molecules, has been refined at a resolution of 1.5 Å to an R-factor of 18.7%. The sequence of LALF, with secondary structural elements indicated, is shown in Figure 1.

Overall structure

The crystal structure of LALF reveals a simple tertiary fold with a striking shape and amphipathicity. The molecule is

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       -4  1             10                  20                  30
LALF:  E A E A D G I W T Q L I F T L V N N L A T L W Q S G D F Q F L D H E C
                 <-----------helix 1------------>      <--strand 1-->   <------

       32             40                  50                  60
LALF:  H Y R I K P T F R R L K W K Y K G K F W C P S W T S I T G R A T K S S
       -----strand 2------>   <--------strand 3--------->   <------strand 4------>

       86                                104
LBP:   R V Q G R W K V R K S F F K L Q G S F
       86                                104
BPI:   K I S G K W K A Q K R F L K M S G N F

PMB:   K' T K' B K' F' - L K' K' T
               |_______________|

                 70                  80                  90                  100
LALF:  R S G A V E H S V R N F V G Q A K S S G L I T Q R Q A E Q F I S Q Y N
         <--------------helix 2----------------->     <---------helix 3--------->
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Fig. 1. Sequence of recombinant LALF with secondary structure elements indicated. The aligned sequences of LBP, BPI and PMB are also shown. For LALF, LBP and BPI, residues marked in large boldface have negative values of hydrophobicity (i.e. hydrophilic) according to Eisenberg's normalized consensus scale. For PMB, the letter K′ is used for diaminobutyric acid (because of its close similarity to lysine); B, the bridging diaminobutyric acid residue, in which the side-chain amino group makes an amide bond to the C-terminal threonine carboxylate; F′, D-phenylalanine; −, an alignment group.

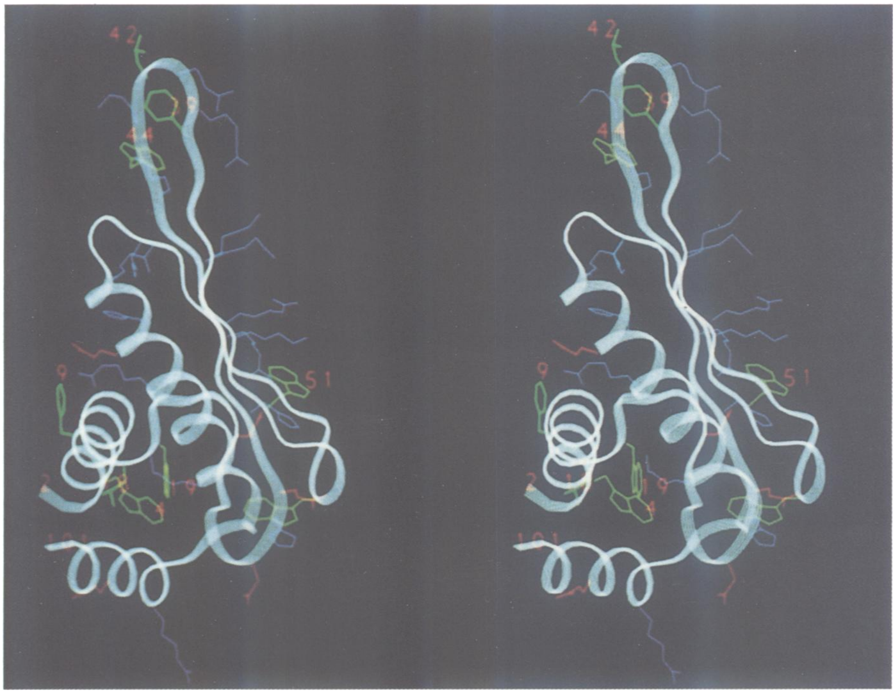

Fig. 2. Stereo ribbon diagram of LALF drawn through the Cα positions, with selected side-chains: all basic residues (Arg, Lys, His) (blue), all tryptophans (yellow), exposed hydrophobic groups (green) and all acidic groups (Asp, Glu) (red).

wedge-shaped, ~40 Å tall and 28 Å wide at its base (Figures 2 and 3). It comprises an N-terminal α-helix that opens into a Π-helix in its final turn, followed by a simple four-stranded anti-parallel β-sheet and two C-terminal α-helices. The three helices form a bundle that packs against the β-sheet and encloses a hydrophobic and highly aromatic core. The N-terminal helix is unusually hydrophobic, with several hydrophobic side-chains exposed to solvent, particularly Phe9 and Leu18. According to Eisenberg's normalized consensus scale (Eisenberg, 1984), helix 1 has a peak hydrophobicity index (HI) of 0.62 and a hydrophobic moment (μ) of 0.18 (for an 11-residue window), placing it in the region of 'multimeric transmembrane' helices. The other two helices have hydropathic and amphipathic characters typical of globular proteins. Strands 2 and 3 of the β-sheet run the length of the molecule, are connected at the bottom by a disulfide bridge (Cys31−Cys52) and at the top extend 12 Å beyond the main body of the protein, forming a positively charged amphipathic hairpin loop with a type I′ turn. The structure contains two salt bridges: the first comprises Asp23, His32 and Arg34, linking strands 1 and 2. The second salt bridge is between Glu72 and Arg76 in helix 2; the phenyl ring of Phe9 is perpendicular to the plane of the carboxylate group and makes an unusual van der Waals contact. His73 sits above the second salt bridge but is not involved in close ionic interactions.

The large outer face of the β-sheet, including one side of the protruding loop, is highly basic, with 10 positively charged residues (lysines and arginines) and an exposed tryptophan (Trp51). The opposite exposed face of the loop

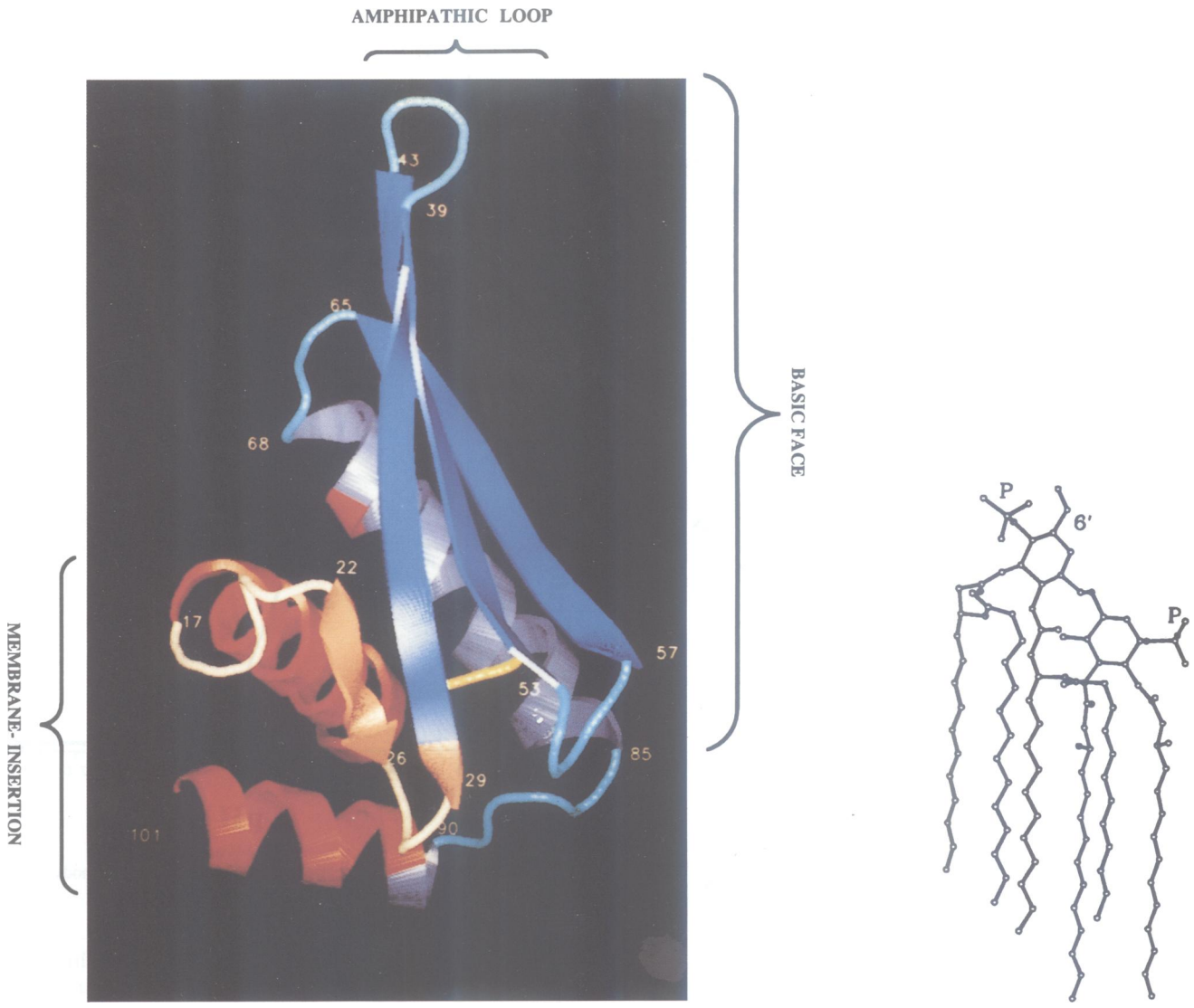

Fig. 3. Schematic ribbons representation of LALF with the ends of secondary structure elements numbered. Helices are shown as corkscrews, strands as arrows, turns as strings and the disulfide bond as a yellow string. The different shades of red (aa 2–30, 92–101 and 72) indicate the negatively charged surfaces of the LALF structure (membrane insertion), the different shades of blue (aa 31–91) the positively charged surfaces (basic face). The amphipathic loop includes amino acids 32–50.

Fig. 4. Model structure of lipid A (Kastowsky *et al.*, 1992) drawn on the same scale as Figure 3. The oligosaccharide (O-antigen, outer core and inner core) portion of LPS is covalently linked to lipid A at its 6′ position. Lipid A is a β,1–6-linked disaccharide of glucosamine that is N- and O-acetylated with R-3-hydroxymyristate at positions 2, 3, 2′ and 3′, and is phosphorylated at positions 1 and 4′ (labelled P). Two lipids are further esterified with laureate and myristate.

is hydrophobic, with Phe39, Leu42 and Trp44 pointing out into solution; this surface is contiguous with a hydrophobic cleft formed by the upper half of helix 1, parts of the inner face of the sheet and parts of helix 2. The surface charge distribution of LALF is bipartite (Figures 2 and 3): the lower left surface contains all six acidic groups and no basic groups (including the N-terminal aspartate and the C-terminal carboxylate; the only exception is the N-terminal amino group). The upper right surface contains only basic groups. At neutral pH, the molecule will thus have a large overall electrostatic dipole.

LPS-binding motif

LALF recognizes the lipid A portion (Figure 4) of individual soluble LPS molecules (Warren *et al.*, 1992), which are obtained below the critical micellar concentration. The simplest molecules that bind lipid A with high affinity are the polymyxin family of antibiotics; these are positively charged amphipathic cyclic oligopeptides linked to a single fatty acid (Morrison and Jacobs, 1976). Sequence similarity (Figure 1) and competitive binding of LALF and polymyxin B (PMB) to LPS (unpublished data), suggest a common LPS-binding epitope in which the cyclic peptide is analogous to part of the amphipathic loop of LALF and binds the phosphoglucosamine portion of lipid A. In support of this notion, it has been shown that LPS binding to LALF reduces tryptophan fluorescence (Wainwright *et al.*, 1990), indicating that a tryptophan becomes buried. LALF has two exposed tryptophans: Trp44 in the amphipathic loop and Trp51 near the bottom of the basic face.

We propose that an analogous amphipathic loop exists for two human proteins that bind lipopolysaccharide: LBP (Schumann *et al.*, 1990) and BPI (Gray *et al.*, 1989), two

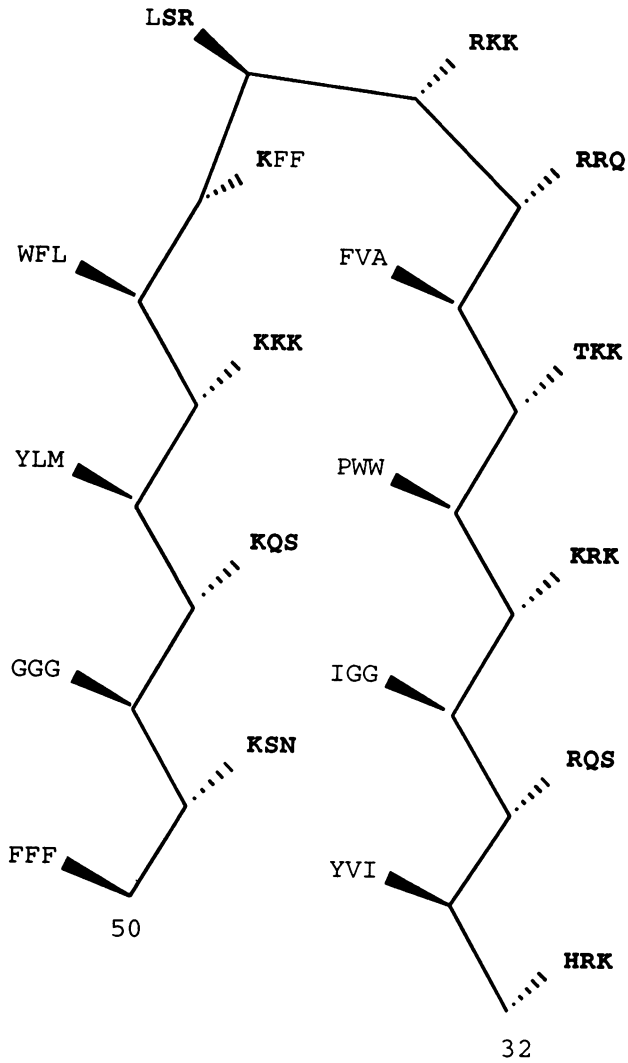

Fig. 5. A schematic of the LALF loop, indicating the direction of side-chains and the putative locations of LBP and BPI. The three letters at each position correspond to LALF, LBP and BPI, in that order. Solid bonds/dashed bonds indicate side-chains pointing out of/into the plane of the diagram. The residues marked in boldface have negative values of hydrophobicity according to Eisenberg's normalized consensus scale.

50 kDa proteins with 44% sequence identity. The loop of LALF is distinguished by an alternating series of positively charged and hydrophobic residues that, by virtue of the extended β-conformation, point in opposite directions, and a single pair of positive charges that, because of the β-turn conformation, point in the same direction and maintain the amphipathicity. Inspection of the LBP and BPI sequences reveals a similar pattern of alternating residues that could produce an amphipathic loop; the 19 residue stretch contains six basic groups and no acidic groups (Figures 1 and 5). Near the top of the loop, one amphipathic pair of residues (Ser/Arg96 and Phe97) is reversed, but it is possible that a different conformation of the hairpin turn would maintain the amphipathicity of the loop in BPI and LBP. To quantify this observation, we used Eisenberg's measure of amphipathicity, the 'hydrophobic moment' (Eisenberg, 1984). Assuming β-conformation for LALF, LBP and BPI, and using an 11-residue search window, the sequence of residues 39–49 for LALF (μ = 0.96) and 84–94 for LBP (μ = 0.92) and BPI (μ = 0.98), had the highest hydrophobic moments in each sequence. Mutational analysis of BPI narrowed LPS-binding region to the first two hundred residues (Gray *et al.*, 1989; Ooi *et al.*, 1991), consistent with our assignment.

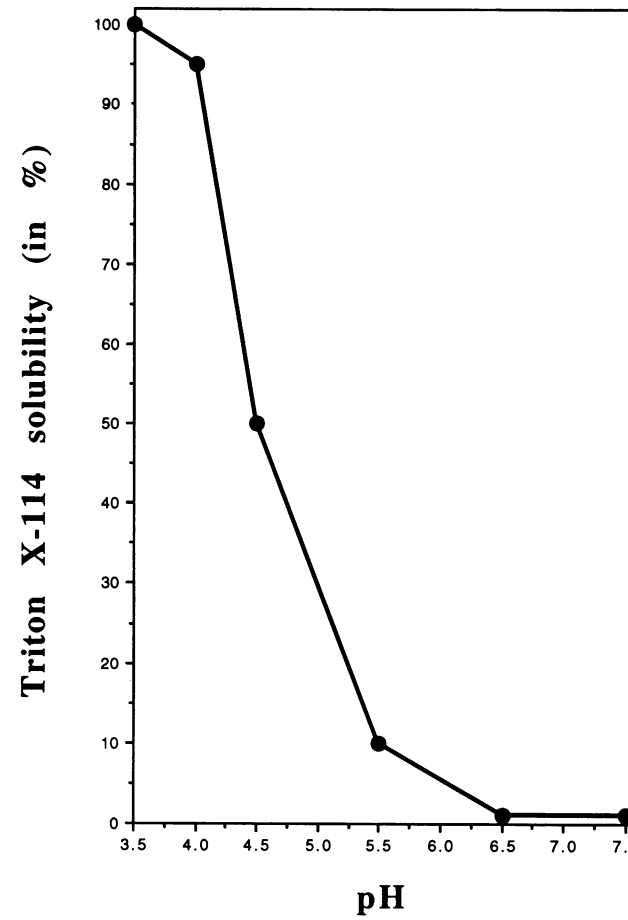

Fig. 6. Detergent partition experiment (see Materials and methods): solubility of LALF in the detergent phase as a function of pH.

Membrane insertion

Two lines of evidence indicate that LALF interacts not only with individual LPS molecules, but also with intact membranes that contain LPS: LALF inhibits the growth of Gram-negative bacteria (Morita *et al.*, 1985) and it can lyse red blood cells once they have been impregnated with LPS (Ohashi *et al.*, 1984). Furthermore, the amphipathic character and charge distribution of LALF have strong parallels with proteins that insert into membranes via a low pH trigger, such as the colicins (Parker *et al.*, 1989) and the bacterial exotoxins (Allured *et al.*, 1986; Chloe *et al.*, 1992). We therefore measured the ability of LALF to partition into the detergent Triton X-114, an assay that has been used to assess membrane insertion of the bacterial exotoxins (Bordier, 1981; Sandvig and Moskaug, 1987). LALF performs similarly: at neutral pH, LALF remains in the aqueous phase, but moves into the detergent phase upon acidification at pH 4.5 (Figure 6). The partitioning is reversible: LALF can be completely reextracted from the detergent phase by raising the pH above 6.0. A pH-induced denaturation can be ruled out since LALF crystallizes

isomorphously between pH 8 and pH 3, and its tryptophan fluorescence is unchanged in the same pH range. Membrane insertion of the bacterial exotoxins (Li, 1992) and colicins (van der Goot *et al.*, 1991) involves protonation of the negatively charged acidic side-chains (pKa for Asp and Glu ~4.2). Protonation of LALF would make its lower left surface uncharged and hydrophobic (Figure 3), creating a molecule that was one large amphiphile. The hydrophobic part could then enter the lipid bilayer, with the basic side-chains pointing out towards the negatively charged head groups of LPS. How does the low pH arise *in vivo*? One possibility comes from studies on the colicins, proteins that insert into the inner membrane of Gram-negative bacteria, even though the periplasmic space is not thought to be acidic. It has been shown in that case that the negatively charged phospholipid head groups create a local pH below the bulk periplasmic pH (van der Goot *et al.*, 1991). A similar effect could provide the trigger for insertion of LALF into the bacterial outer membrane.

Prospects for drug design

Therapeutic strategies for the treatment of septic shock in humans have focused on the neutralization of LPS and its endogenous mediators. The medical uses of LPS-binding drugs, such as PMB or monoclonal antibodies (Cohen and Glauser, 1991; Ziegler *et al.*, 1991) are limited by toxic side effects in the former case (Craig *et al.*, 1974) or by uncertainties regarding mode of action in the latter (Cohen and Glauser, 1991). Recombinant LALF may be a useful therapeutic agent in humans, as it does not show toxic side effects (Alpert *et al.*, 1992) and LALF is active in animal models with peritoneal sepsis even when administered after bacterial infection (Siber *et al.*, 1993), while other drugs show effects only when given preventively (Levin *et al.*, 1987). Our structure can be used in the design of conformationally stabilized peptides with therapeutic properties in neutralizing endotoxin. Synthetic peptides based on the structure of PMB, which we suggest are similar to part of the LALF loop structure, have already been shown to bind LPS with high affinity and reduce mortality in animals (Rustici *et al.*, 1993). The use of peptides derived from the human LBP and BPI loop sequences may have the advantages of improved affinity, spectrum of binding and pharmacology.

Materials and methods

Purification and crystallization

LALF was expressed in yeast and purified as described by Wainwright *et al.* (1990). The recombinant protein contains four additional residues at the N terminus (Glu-Ala-Glu-Ala) not present in the native protein and an authentic C-terminus. It was crystallized by equilibration with 1.1 M citrate, pH 8.5, at 4°C. The crystals belong to space group $P2_1$ with cell dimensions a = 52.8 Å, b = 39.9 Å, c = 26.3 Å and β = 98.6°, and have a single molecule in the asymmetric unit.

Crystallographic analysis

Data were collected using the San Diego-style multiwire area detector on an Rigaku rotating anode X-ray source equipped with a graphite monochromator (16 318 unique reflections to 1.5 Å; R_{merge} = 3.0%; completeness, 93.8%). Derivatives were obtained by soaking native crystals with 0.5 mM K_2PtBr_6 for 14 days (6999 unique reflections to 2.0 Å; R_{merge} = 3.0%; R_{deriv} = 21.0%; completeness, 92.7%) or 0.25 mM CH_3HgNO_3 for 2 days (7033 unique reflections to 2.0 Å; R_{merge} = 4.2%; R_{deriv} = 14.0%; completeness, 94.1%). Heavy atom sites were found from difference Pattersons and cross-difference Fouriers (one site for K_2PtBr_6, two sites for CH_3HgNO_3) and refined with the program HEAVY, using origin-removed Patterson coefficients (Terwilliger and Eisenberg, 1983). The amino acid sequence of LALF was fitted to an electron density map calculated with unmodified MIR phases, using the anomalous and isomorphous data from both derivatives [mean figure of merit (FOM) to 2.0 Å, FOM = 0.57]. The model was improved by model building (Jones, 1978) and conventional least-squares refinement (Konnert and Hendrickson, 1980). The final R factor at 1.5 Å resolution is 18.7% (all data between 10–1.5 Å). The r.m.s. deviation from ideal values for bond lengths is 0.017 Å and for bond angles is 2.7°.

$$R_{merge} = \sum\sum | I_i - \langle I \rangle | / \sum I_i$$

$$R_{deriv} = \sum | F_{PH} - F_P | / \sum | F_P |$$

Calculation of hydrophobic moment

The hydrophobic moment (μ) is defined as the vector sum of hydrophobic indices (HI_n) over N residues (Eisenberg, 1984). For an α-helix, in which successive side-chains emerge from the helix at 100° intervals, it is given by:

$$\mu = 1/N\{[\sum HI_n \sin(n \times 100°)]^2 + [\sum HI_n \cos(n \times 100°)]^2\}^{1/2}$$

and for a β-strand, in which successive residues differ by 180°, it is given by:

$$\mu = 1/N \, | \sum HI_n \cos(n \times 180°) |$$

Detergent partition experiment

This experiment is based on the observation that the detergent Triton X-114 forms a single phase with water at 4°C, but separates into two phases on warming to 37°C (Madshus and Collier, 1989). 5 g of Triton X-114 were dissolved in 100 ml of 20 mM sodium phosphate (pH 7.5) and 140 mM NaCl at 4°C. Recombinant LALF (2 μg) was added to 50 μl of the solution and the pH adjusted with citrate. The samples were incubated at 37°C for 30 min, until two phases appeared and then centrifuged for 3 min. The upper aqueous phase was applied onto an SDS–acrylamide gel and the stained gel analyzed densitometrically.

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